

**UNITED STATES PATENT APPLICATION**

entitled

**SPRAY AND/OR SOIL TREATMENT OF TOBACCO TO REDUCE TSNA<sub>s</sub>**

by

**Qinglin Li  
11601 Aprilbud Drive  
Richmond, Virginia 23229  
CITIZENSHIP CANADA**

**Walter Hempfling  
9183 Madison Leigh Court  
Mechanicsville, Virginia 23111  
CITIZENSHIP USA**

and

**Marc Robert Krauss  
13814 Beechwood Point Circle  
Midlothian, Virginia 23112  
CITIZENSHIP USA**

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BURNS, DOANE, SWECKER & MATHIS, L.L.P.  
Post Office Box 1404  
Alexandria, Virginia 22313-1404  
(703) 836-6620**

## SPRAY AND/OR SOIL TREATMENT OF TOBACCO TO REDUCE TSNAs

### CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Serial No. 60/443,848 filed on January 31, 2003, which is herein incorporated by reference in its entirety for all purposes.

### FIELD OF INVENTION

The invention relates to methods for increasing the concentration of advantageous antioxidants in tobacco leaves by spraying at least one chemical solution onto a growing tobacco plant and/or soil treatment of growing tobacco plants. The application of the chemical solution preferably occurs between topping and harvest, and can be optimized to stimulate the production of antioxidants in tobacco leaves which interfere with the formation of TSNAs during curing of the tobacco leaves.

### BACKGROUND OF THE INVENTION

Tobacco-specific nitrosamines (TSNAs), such as N-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), can be found in smokeless tobacco; mainstream smoke; and side stream smoke of cigarettes. It has been reported that air-cured and flue-cured tobacco contain tobacco-specific nitrosamines. See, "Effect of Air-Curing on the Chemical Composition of Tobacco", Anna Wiernik *et al.*, *Recent Adv. Tob. Sci.*, (1995), 21: 39-80. According to Wiernik *et al.*, TSNAs are not present in significant quantities in growing tobacco plants or fresh cut tobacco (green tobacco), but are formed during the curing process. Bacterial populations which reside on the tobacco leaves are stated to largely cause the formation of nitrites from nitrate during curing and possibly effect the direct catalysis of the nitrosation of secondary amines at physiological pH values. The affected secondary amines include tobacco alkaloids, which form TSNAs when nitrosated.

Because curing of tobacco leaves is normally performed by the farmer who grows the tobacco, a simple, economical and non-labor-intensive method of reducing TSNA levels in the cured tobacco leaves is desirable.

### SUMMARY OF THE INVENTION

5           According to one embodiment, a method of reducing tobacco-specific nitrosamines in cured tobacco, comprises raising the levels of antioxidants in tobacco prior to being cured, wherein the levels of antioxidants are raised by spraying a chemical solution onto an aerial part (*i.e.*, that part of the plant growing above ground) of a tobacco plant prior to  
10           harvesting and wherein the chemical solution effects an increase in the level of antioxidants in the tobacco plant. The chemical solution preferably includes a plant growth hormone such as abscisic acid (ABA) or analog thereof, a plant activator such as salicylic acid (SA) (commercially available as ACTIGARD®) or analog thereof, a plant activator such as a harpin protein containing product commercially available as MESSENGER®, a herbicide such as methyl viologen (MV) or analog thereof, a stress inducing agent such as hydrogen  
15           peroxide, sodium chloride (NaCl), or sulfur dioxide, or combinations thereof. Preferably, the analog of MV is Paraquat (a quaternary nitrogen herbicide widely used for weed control). Preferably, the tobacco plants are sprayed with the chemical solution in a single application or multiple applications about 1-3 weeks prior to harvest. Preferably, the tobacco is burley tobacco and the method further includes air curing the burley tobacco.  
20           The tobacco is preferably incorporated in a cigarette.

          According to another embodiment, a method of reducing tobacco-specific nitrosamines in cured tobacco, comprises raising the levels of antioxidants in tobacco prior to being cured, wherein the levels of antioxidants are raised by soil treatment of tobacco plants prior to harvesting and wherein the soil treatment effects an increase in the level of  
25           antioxidants in the tobacco plant. The soil can be treated with a chemical solution which preferably includes a plant growth hormone such as abscisic acid (ABA) or analog thereof, a plant activator such as salicylic acid (SA) (commercially available as ACTIGARD®) or analog thereof, a plant activator such as a harpin protein containing product commercially

available as MESSENGER®, a herbicide such as methyl viologen (MV) or analog thereof, a stress inducing agent such as hydrogen peroxide, sodium chloride, or sulfur dioxide, or combinations thereof. Preferably, the analog of MV is Paraquat (a quaternary nitrogen herbicide widely used for weed control). Preferably, the soil is treated in a single application or multiple applications about 1-3 weeks prior to harvest. Preferably, the tobacco is burley tobacco and the method further includes air curing the burley tobacco. The tobacco is preferably incorporated in a cigarette.

### **BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1 depicts a graph of antioxidant capacity of green and cured Oriental, bright and burley tobacco grown without a spray treatment to raise the antioxidant capacity.

Figure 2 depicts a mechanism by which antioxidants in tobacco inhibit TSNA formation.

Figure 3 depicts a graph of the effects on antioxidant capacity of spraying salicylic acid and methyl viologen onto greenhouse tobacco.

### **DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS OF THE INVENTION**

The amount of tobacco specific nitrosamines (TSNAs) in cured tobacco leaves can be reduced by spraying growing tobacco plants with a chemical solution and/or treating the soil surrounding roots of growing tobacco plants with a chemical solution. In particular, TSNAs in cured tobacco leaves may be reduced by raising the levels of antioxidants in the tobacco leaves prior to harvesting the plants.

The antioxidant levels in tobacco leaves sprayed with the chemical solution are preferably raised at least 25%, preferably at least 30%, and more preferably at least 50% compared to tobacco leaves of untreated plants grown without the spray treatment. Likewise, the antioxidant levels in tobacco leaves of tobacco plants grown in soil treated with the chemical solution are preferably raised at least 25%, preferably at least 30%, and more preferably at least 50% compared to tobacco leaves of untreated plants which without

the soil treatment. Figure 1 shows an example of the antioxidant capacity of green and cured Oriental, bright and burley tobacco obtained from untreated plants.

It is believed that TSNA's are formed predominantly during the curing process. While not wishing to be bound by theory, it is believed that the amount of TSNA's in cured tobacco leaves results from the reaction of a reactive nitrosating species with the tobacco alkaloids.

The amount of antioxidants in tobacco leaves during the time of air-curing is believed to be advantageous to the inhibition of TSNA formation. An elevated concentration of native antioxidants during senescence and air-curing of tobacco can be obtained by spraying of a chemical solution preferably comprising an aqueous solution containing one or more chemical compounds onto the growing tobacco plant and/or treating the soil surrounding roots of growing tobacco plants with the aqueous solution. For example, it is possible to increase foliar contents of native antioxidants by stressing the tobacco plant, *e.g.*, using ABA to reduce the availability of carbon dioxide necessary for photosynthesis. Preferably the tobacco is burley tobacco. The spraying of the solution and/or soil treatment preferably occurs between topping (*i.e.*, removal of the flower from the tobacco plant to stimulate leaf production) and harvest, and is intended to stimulate the production of antioxidants and interfere with the formation TSNA's during curing of the tobacco. Preferably, the level of antioxidants is increased to an amount sufficient to prevent significant nitrosation during the yellowing and browning phases of curing. The chemical spraying and/or soil treatment can be carried out only once or the tobacco plants can be sprayed and/or subjected to soil treatment more than once. For example, the plants can be sprayed at layby (when the plants are about knee-high) and the spray treatment can be repeated periodically such as every 5 to 15 days until harvest. Likewise, the soil in which the plants are grown can be treated at layby and the soil treatment can be repeated periodically such as every 5 to 15 days until harvest.

The spray and/or soil treatment can be combined with other methods of elevating tobacco leaf antioxidants to a level that inhibits nitrosation. For example, the antioxidants can be raised by pruning of the tobacco plants (*e.g.*, root pruning and xylem cutting) prior

to harvest. Thus, root pruning and/or xylem cutting may be used to inhibit TSNA formation during air-curing. Under the stressful conditions caused by root pruning and xylem cutting, the plant's capacity to assimilate CO<sub>2</sub> is reduced, and the photosynthetic electron flux to O<sub>2</sub> will increase resulting in the increased production of superoxide and hydroxyl radicals. These molecules induce antioxidants to cope with the oxidative stress. (See commonly-owned U.S. Patent Application No. 10/235,636, filed on September 6, 2002, which is incorporated by reference in its entirety herein).

Disturbances in photosynthetic activity can be used to cause the formation of reactive oxygen species. Thus, chemicals that directly affect chloroplast activity can stimulate processes that induce formation of reactive oxygen species. Redox-active herbicides such as the diphenyl ethers (*e.g.*, acifluorfen) can act through the production of reactive oxygen. Such herbicides can cause the activity of antioxidants such as ascorbate, glutathione and glutathione reductase to increase while the plant is actively defending itself against the herbicide. Bipyrindyl herbicides, such as Paraquat (also known as methyl viologen) and Diquat, can be used to increase oxidative stress directly by generating reactive oxygen radicals.

Growing tobacco plants can be sprayed with a chemical solution, preferably an aqueous solution, which effects an increase in antioxidant levels in the tobacco leaves and thus reduced TSNA's during curing of the tobacco leaves. Likewise, the soil of tobacco plants can be treated with a chemical solution, preferably an aqueous solution, which effects an increase in antioxidant levels in the tobacco leaves and thus reduced TSNA's during curing of the tobacco leaves. Preferably, a chemical solution containing at least one chemical compound is sprayed onto growing tobacco plants using farm equipment suitable for spray application of liquids to tobacco plants. If desired, spraying of such compounds can be combined with other nitrosamine-reducing treatments such as root pruning and xylem cutting, to result in tobacco leaves with exceptionally low nitrosamine contents during the curing process. Further, the soil surrounding the roots of tobacco plants can be sprayed and/or irrigated using a chemical solution which effects the desired increase in the antioxidant levels in the tobacco leaves.

1. Definitions and Abbreviations

1.1 Definitions

By the term "herbicide" is meant to include reagents that increase antioxidant activity. For example, preferred herbicides include but are not limited to redox-active herbicides and bipyridyl herbicides. Preferred redox-active herbicides include diphenyl ethers (*e.g.*, acifluorfen and acifluorfen sodium). Preferred bipyridyl herbicides include methyl viologen (*e.g.*, Paraquat) and Diquat.

By the term "plant growth hormone" is meant to include those plant growth hormones which modulate the antioxidant level and thereby TSNA in the tobacco plant.

Preferred plant growth hormones are abscisic acid (ABA) and jasmonic acid.

By the term "plant activator" is meant to include reagents which modulate the antioxidant level and thereby TSNA in tobacco plants. A preferred plant activator is a salicylic acid, or an analog of salicylic acid, (*e.g.*, ACTIGARD®), or a harpin protein (*e.g.*, MESSENGER®).

By the term "stress inducing reagents" is meant to include reagents which modulate the antioxidant level and thereby TSNA in tobacco. Preferred stress inducing reagents include sulfur dioxide, sodium chloride and hydrogen peroxide.

1.2 Abbreviations

ABA	abscisic acid
FRAP	Ferric-Reducing Antioxidant Power
HPLC	high performance liquid chromatography
MV	methyl viologen
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
NNN	N-nitrosornicotine
SA	salicylic acid
TSNA	tobacco-specific nitrosamines

2. Herbicides

One embodiment comprises spraying an effective amount of at least one herbicide such as methyl viologen (MV) or one or more analogs thereof (*e.g.*, Paraquat), or Diquat (chemical name: 1',1'-ethylene-2,2'-bipyridylium) and/or other herbicides, to the aerial part of topped tobacco plants several weeks or days before harvesting. Also contemplated are diphenyl ethers. Preferred diphenyl ethers include but are not limited to acifluorfen and acifluorfen sodium, which respectively have the chemical names of

5-[2-chloro-4-(trifluoro-methyl)phenoxy]-2-nitrobenzoate and sodium 5-[2-chloro-4-(trifluoro-methyl)phenoxy]-2-nitrobenzoate. Preferably, the spraying is performed one or more times in the field about 1-3 weeks before harvesting (*i.e.*, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 days before harvesting). It has been found that such spraying results in a significant reduction of TSNA's during air-curing of burley tobacco. The reduction of TSNA's during curing may be accompanied by an increase in total antioxidant activity, as measured by the Ferric-Reducing Antioxidant Power assay ("FRAP").

Another embodiment comprises applying an effective amount of at least one herbicide such as methyl viologen (MV) or one or more analogs thereof, such as Paraquat (chemical name: 1,1'-dimethyl-4,4'-bipyridinium), and/or other herbicides, to the soil surrounding roots of topped tobacco plants several weeks or days before harvesting. Preferably, the soil treatment is performed one or more times in the field about 1-3 weeks before harvesting (*i.e.*, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 days before harvesting).

The herbicide(s) can be applied in a concentration of about 0.01 mM to about 0.3 mM, more preferably 0.05 to about 0.15 mM and most preferably in an amount of about 0.08 to about 0.11 mM (*e.g.*, about 0.1 mM Paraquat) (and every 0.01 mM unit inbetween these ranges). Typically about 50 mL to about 500 mL of the solution is sprayed per plant, more preferably an amount of about 100 mL to about 300 mL and most preferably about 150 mL to about 250 mL of solution is sprayed onto the leaves or the soil of each plant.



3. Plant Activators

Another embodiment comprises spraying an effective amount of a plant activator, such as salicylic acid (SA), or one or more analogs thereof, preferably ACTIGARD® (active ingredient chemical name: 1,2,3-benzothiadiazole-7-thiocarboxylic acid-5-methyl-ester), or a harpin protein such as MESSENGER® (harpin, HrpN from *Erwinia amylovora*; GenBank Accession No: AAC31644), to, for example, the aerial part of topped tobacco plants several weeks or days before harvesting. ACTIGARD® is manufactured by Syngenta Crop Protection, Greensboro, North Carolina. MESSENGER™ is available from Eden Bioscience Corporation, Bothell, Washington. Preferably, the spraying is performed one or more times in the field about 1-3 weeks before harvesting (*i.e.*, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 days before harvesting). For example, ACTIGARD® can be sprayed onto the tobacco plants in a single application at a concentration of about 1 ounce (oz) per acre about 1 week prior to harvest or in a series of applications at the same or different concentrations, *e.g.*, 1 oz/acre, 0.5 oz/acre and/or 0.25 oz/acre. MESSENGER® can be sprayed onto the tobacco plants in a single application at a concentration of 9 ounces per acre or in a series of applications at the same or different concentrations. ACTIGARD® is believed to disable enzyme activity thereby creating oxidative stress to which the tobacco plant responds by production of antioxidants. MESSENGER® is believed to create a plant response known as systemic acquired resistance that manifests a production of antioxidants. Due to the increase in antioxidant level in the treated tobacco leaves, a significant reduction of TSNA's can be obtained during curing of the tobacco leaves.

A further embodiment comprises applying an effective amount of salicylic acid (SA), or one or more analogs thereof, preferably ACTIGARD®, or a harpin protein such as MESSENGER®, to the soil surrounding roots of topped plants several weeks or days before harvesting. Preferably, the soil treatment is performed one or more times in the field about 1-3 weeks before harvesting (*i.e.*, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 days before harvesting).

Salicylic acid and its analogs such as ACTIGARD® can be used to treat plants in concentrations of about 0.1 mM to about 10 mM, or more preferably about 0.5 mM to about 2 mM and most preferably in an amount of about 0.8 mM to about 1.2 mM. Typically about 50 mL to about 500 mL of the solution is sprayed per plant, more preferably an amount of about 100 mL to about 300 mL and most preferably about 150 mL to about 250 mL of solution is sprayed onto the leaves of each plant or administered onto the soil surrounding the roots of each plant.

A harpin protein such as MESSENGER® can be used to treat the leaves or roots of the plants in an amount of about 4.5 oz/acre to about 9 oz/acre (9 oz/acre is the FDA approved amount).

#### 4. Plant Growth Hormones

Another embodiment of the present invention comprises spraying an effective amount of at least one plant growth hormone such as abscisic acid (ABA), or one or more of its more stable analogs, or jasmonic acid or one or more analogs thereof preferably onto the aerial part of topped tobacco plants several weeks or days before harvesting. Preferably, the spraying is performed one or more times in the field about 1-3 weeks before harvesting (*i.e.*, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 days before harvesting). Due to the increase in antioxidant level in the treated tobacco leaves, a significant reduction of TSNAs can be obtained during curing of the tobacco leaves.

ABA is a major controller of stomatal closing, and naturally increases in concentration in tobacco leaves as a response to water-deficit stress. See Figure 2. The CO<sub>2</sub> supply for photosynthesis can be interrupted leading to enhancement of the native leaf antioxidants. A solution of ABA, or a suitable analog, can be sprayed onto the tobacco plant such that stomata remain at least partially closed for a short time period, but then open again to support photosynthesis, akin to the action of root pruning, but without loss of turgor due to water deficit. The effects of spraying ABA on growing tobacco plants as compared to MV is shown in Figure 3. When administering ABA onto the leaves of the

plant or on the soil surrounding the roots of the tobacco plant, preferably an amount of about 0.05 mM to about 1 mM ABA is used, more preferably about 0.1 to about 0.5 mM ABA and most preferably about 0.2 mM to about 0.4 mM ABA.

5 Jasmonic acid is preferably administered to the leaves or the soil surrounding the roots of the tobacco plants in an amount of about 0.01 mM to about 2.0 mM and more preferably in an amount of about 0.1 mM to about 1 mM.

10 A further embodiment of the present invention comprises applying an effective amount of at least one plant growth hormone such as abscisic acid (ABA), or one or more of its more stable analogs or jasmonic acid or one or more analogs thereof onto the soil surrounding roots of topped tobacco plants several weeks or days before harvesting. Preferably, the soil treatment is performed one or more times in the field about 1-3 weeks before harvesting (*i.e.*, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 days before harvesting).

15 Typically about 50 mL to about 500 mL of the solution is sprayed per plant, more preferably an amount of about 100 mL to about 300 mL and most preferably about 150 mL to about 250 mL of solution is sprayed onto each plant (*e.g.*, the leaves or the soil surrounding the roots).

#### 5. Stress Inducing Agent

20 Another embodiment of the present invention comprises spraying a solution containing an effective amount of at least one stress inducing agent such as hydrogen peroxide, NaCl (salt) or sulfur dioxide onto the aerial part (the leaves) of topped tobacco plants several weeks or days before harvesting. Preferably, the spraying is performed one or more times in the field about 1-3 weeks before harvesting (*i.e.*, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 days before harvesting).

25 A further embodiment of the present invention comprises applying a solution containing an effective amount of at least one stress inducing agent such as hydrogen peroxide, a salt (*e.g.*, NaCl) or sulfur dioxide onto the soil surrounding the roots of topped tobacco plants several weeks or days before harvesting. Preferably, the soil treatment is

performed one or more times in the field about 1-3 weeks before harvesting (*i.e.*, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 days before harvesting).

5 The foregoing methods of soil treatment and spraying one or more chemical compounds onto the leaves of the tobacco plants before harvesting can be combined with other methods of increasing antioxidants, to reduce nitrosamines in tobacco plants during the curing process. For example, partial removal of the root structure (root pruning) of burley tobacco in the field, about a week before harvesting, results in significant reduction of tobacco-specific nitrosamines during air-curing, probably due to the resulting increase in  
10 total antioxidant capacity (measured by the Ferric-Reducing Antioxidant Power assay) that persists during the curing process.

Hydrogen peroxide treatment is preferably applied as a solution containing hydrogen peroxide in a range of about 1% to about 5%, more preferably in a range of about 2% to about 4%, and most preferably in a range of about 2.5% to about 3.5% being  
15 sprayed on the leaves and/or onto the soil surrounding the roots.

NaCl is administered to the plant by spraying on the leaves or onto the soil surrounding the roots a solution of sodium chloride comprising about 0.8% to 0.1% sodium chloride, more preferably from about 0.6% to about 0.2% NaCl, and most preferably 0.5% to about 0.3% (*e.g.*, about 0.4%) NaCl.

20 The sulfur dioxide is administered to the plant in an amount of about 50 nL/L to about 500 nL/L, more preferably in an amount of about 100 nL/L to about 250 nL/L and most preferably in an amount of about 150 nL/L to about 200 nL/L.

Typically about 50 to about 500 mL of a solution with one or more of these reagents is sprayed per plant, more preferably an amount of about 100 to about 300 mL and most  
25 preferably about 150 to about 250 mL of solution is sprayed onto each plant.

#### 6. Combinations of Reagents

Also contemplated herein are combinations of reagents. For example, one or more herbicides, stress inducing reagents, plant growth hormones, and plant activators can be

combined either in an admixture or applied separately. The combination of reagents can be used to treat the tobacco such that the tobacco has increased antioxidant activity and/or production of antioxidants, which correspondingly reduces TSNAs. Combinations of such reagents include a plant growth hormone in combination with a plant activator (*e.g.*, a salicylic acid combined with jasmonic acid). Additional combinations include jasmonic acid in combination with one or more stress reducing reagents, one or more herbicides, one or more plant activators, or with a different plant growth hormone. When used in combinations, preferably the combinations of reagents may require less of each reagent to be administered to the tobacco plant than if the reagent was administered alone (*i.e.*, not in combination with other antioxidant inducing reagents).

Such combinations can be administered onto the aerial part of a tobacco plant (topped or prior to being topped). The combination of reagents can be administered several weeks or days before harvesting, or both. Preferably, the combination of reagents are applied by spraying. Such spraying can be performed one or more times in the field. Preferably, the spraying of the combination of reagents occurs about 1-3 weeks before harvesting (*i.e.*, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 days before harvesting). The combination of reagents can also be administered to the plant, or portions of the plant (*e.g.*, leaves, stems), after the plant has been harvested.

A further embodiment of the present invention comprises applying a solution containing an effective amount of a combination of reagents onto the soil surrounding the roots of a tobacco plants (topped or prior to topping). The solution can be applied several weeks or days before harvesting the plant. Preferably, the soil treatment is performed one or more times in the field about 1-3 weeks before harvesting (*i.e.*, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 days before harvesting). The solution can also be administered to the plant after the plant has been harvested. Typically about 50 to about 500 mL of the solution is sprayed per plant, more preferably an amount of about 100 to about 300 mL and most preferably about 150 to about 250 mL of solution is sprayed onto each plant.

7. Combination of Reagents and Methods for Reducing TSNAs

The reagents discussed above can be used alone to reduce TSNAs or in combination with one another, or in combination with methods which reduce TSNAs. Thus, the methods of treating the soil surrounding the roots or the leaves of the plants with one or more chemical compounds before harvesting can be combined with other methods of increasing antioxidants, to reduce nitrosamines in tobacco plants during the curing process. For example, partial removal of the root structure (root pruning) or xylem cutting of burley tobacco in the field, about a week before harvesting, results in significant reduction of tobacco-specific nitrosamines during air-curing, probably due to the resulting increase in total antioxidant capacity (measured by the Ferric-Reducing Antioxidant Power assay) that persists during the curing process.

The root pruning (or other mechanical stress which produces a similar result) induces water-deficit stress which is believed to result in temporary stomatal closure to preserve water in the plant. Such closure of the stomata also shuts off the supply of atmospheric carbon dioxide that supports photosynthetic carbon dioxide fixation and allows the plant to produce more antioxidants. Other forms of mechanical stress which yield a similar result (*i.e.*, induce production of antioxidants such as xylem cutting) can also be used in combination with any one or combination of the reagents discussed above.

Example 1

TSNA Inhibition Using Salicylic Acid or Methyl Viologen

Topped greenhouse tobacco plants were sprayed with aqueous solutions of salicylic acid, methyl viologen and a control substance, in order to measure the total antioxidant capacity ( $\mu\text{mol/g}$ ) over 24 hours. Mature burley tobaccos (with about 20-22 leaves per plant) were topped two weeks prior to being sprayed with either salicylic acid or methyl viologen. An aqueous solution of salicylic acid (99%; Aldrich Chemical Co.) at 1 M and methyl viologen (98%; Aldrich Chemical Co.) at 0.1 mM concentration were sprayed upon the tobacco leaves. Each plant was sprayed with at least 200 mL of solution with good coverage of each leaf to produce a more uniform response to the chemical spraying. Green

samples were taken 24 hours after spraying. Leaves were checked after sampling to make sure all leaves are representative of the plant conditions. Samples were quickly separated into lamina and midribs as known in the art. Only the lamina was retained for analysis. Sampled green lamina was flash-frozen in liquid nitrogen. The samples can be stored at -80°C if analysis cannot be performed immediately.

Total antioxidant capacity was determined by FRAP assay, and the values were expressed as  $\mu\text{mol/g}$  of fresh weight. The concentration of chlorogenic acid and rutin, two water soluble antioxidants, were measured by HPLC and their contribution to the total antioxidant capacity (measured by FRAP) was calculated. The results (Table 1) show that both methyl viologen and salicylic acid increase antioxidant capacity.

**Table 1**

	Total Antioxidant Capacity ( $\mu\text{mol/g}$ )	Antioxidant Capacity of Chlorogenic Acid ( $\mu\text{mol/g}$ )	Antioxidant Capacity of Rutin ( $\mu\text{mol/g}$ )
Control	40	12	1
Salicylic Acid	50	25	1
Methyl Viologen	55	28	1

The FRAP assay was performed as follows. The green lamina were accurately weighed and extracted with 10% methanol and 0.2 M perchloric acid in water. The mixture was shaken at 4°C on an orbital shaker for an hour. The extracts were then centrifuged at 5,000 rpm for 20 minutes. The supernatant was filtered through Whatman Autovial 0.45  $\mu\text{m}$  PVDF syringe filters and was ready for analysis after appropriate dilution with extraction solution (10% MeOH and 0.2 M perchloric acid in water).

The procedures for the FRAP assay on tobacco samples were modified from that previously described by Benzie and Strain, *Meth. Enzymology* 299: 15-27 (1999). The assay was done manually on a spectrophotometer at room temperature. The FRAP reagent was prepared by combining 300 mM acetate buffer (pH 3.6), 10 mM 2,4,5-tripyridyl-s-

triazine in 40 mM HCl and 20 mM FeCl<sub>3</sub> in the ratio of 10:1:1 (v:v:v). A 100  $\mu$ L aliquot of the sample extraction was added to 3 mL of FRAP reagent and mixed. After the mixture stood at room temperature for 6 min., the absorbance at 593 nm was determined against the FRAP reagent alone. Calibration was against a standard curve (*i.e.*, 500, 1000 and 2000  $\mu$ M ferrous iron) produced by the addition of freshly prepared ferrous sulfate. FRAP values were calculated as micromolar ferrous ion (ferric reducing power) from two determinations.

Chlorogenic acid (*i.e.*, 3-O-caffeoylquinic acid) and rutin (*i.e.*, quercetin 3-rhamnosidoglucoside) are two antioxidants that are water soluble and can easily be measured in plants. Chlorogenic acid and rutin concentrations were determined by high performance liquid chromatographic (HPLC) analysis. Other polyphenols such as the isomers of chlorogenic acid (*i.e.*, 4-O-, and 5-O-caffeoylquinic acid) and scopoletin (*i.e.*, 6-methoxy-7-hydroxycoumarin) were also separated by reverse phase HPLC using a gradient of methanol with 1% glacial acetic acid. 500 mg of tobacco was extracted with 10% methanol and 0.2 M perchloric acid in water. The supernatant was neutralized using K<sub>2</sub>CO<sub>3</sub> to a pH of 6.5 before the sample was injected into the HPLC. Umbelliferone (*i.e.*, 7-hydroxycoumarin) was used as an internal standard and detection was by UV absorption at 326 nm.

### Example 2

The following table (Table 2) shows the results of experiments using an aqueous chemical spray to raise burley native leaf antioxidant capacity in order to interfere with TSNA production during air curing. Burley tobacco plants were grown using standard agronomic practices. ACTIGARD® and MESSENGER® were sprayed on the plants after layby in two-week intervals with a total of five (5) applications of the reagents until the plants were harvested. Treatment with the ACTIGARD® or MESSENGER® at this time in plant growth is not suitable for optimum results for the manufacturer recommended uses of those reagents as plant activators.



At harvest, care was taken to ensure clean tobacco by minimizing contact with the soil. Plants were stalk cut and five stalks were speared on each stick for standard curing in conventional air-curing barns. All treated and control tobaccos were hung with even spacing of sticks. The treated (non-control) tobaccos were surrounded by clean filler tobacco to help to create normal curing conditions. At least three replicate samples were taken at each sampling point from five different plants with 3 leaves from the top one-third of the plant (excluding the top 4 leaves). Each plant was sampled only once. Midveins were separated from the lamina. Both lamina and midveins were weighed and placed in sample bags stored at  $-80^{\circ}\text{C}$ . After freeze-drying, samples were re-weighed to determine the moisture content. Samples were ground to pass through a 40-mesh screen and stored at  $-40^{\circ}\text{C}$  until analysis.

FRAP analysis was performed as follows. Freeze-dried samples were accurately weighed and extracted with 10% methanol and 0.2 M perchloric acid in water. The mixture was shaken at  $4^{\circ}\text{C}$  on an orbital shaker for an hour. The extracts were then centrifuged at 5,000 rpm for 20 minutes. The centrifuged supernatants were then filtered through Whatman Autovial 0.45  $\mu\text{m}$  PVDF Syringe filters. The filtered material was then diluted as discussed in Example 1 above. The FRAP assay was conducted as described by Benzie and Strain (1999) with the following modifications. The FRAP reagent was prepared daily by combining 300 mM acetate buffer (pH 3.6), 10 mM 2,4,5-tripyridyl-s-triazine in 40 mM HCl and 20 mM  $\text{FeCl}_3$  in a ratio of 10:1:1 (v:v:v). A 100  $\mu\text{L}$  aliquot of the sample extraction was added to 3,000  $\mu\text{L}$  (3 mL) of FRAP reagent and mixed. After the mixture stood at room temperature for 6 minutes, the absorbance at 593 nm was determined against the FRAP reagent. Calibration was against a standard curve (*i.e.*, 500, 1000 and 2000  $\mu\text{M}$  ferrous ion) produced by the addition of freshly prepared ferrous sulfate. FRAP values were calculated as micromolar ferrous ion (ferric reducing power) from two determinations. The total antioxidant capacity values are expressed in  $\mu\text{mol/g}$  of fresh weight.

The treated tobaccos resulted in cured leaves with nearly 30% antioxidant capacity increases and reduction of TSNA of about 65-70% as compared to untreated control samples.

Table 2

		Control	MESSENGER®	ACTIGARD®	
		No Spray	9 oz/acre	0.25 oz/acre	0.5 oz/acre
Total Antioxidant Capacity ( $\mu\text{mol/g}$ )		55.4	70.9	71.9	59.1
TSNA	Lamina	3040	1443	1787	1037
TSNA	Mid-rib	5110	1057	1624	1627

Example 3

NaCl Induced Inhibition of TSNA

In tobacco plants, antioxidants interfere with the nitrosation of secondary alkaloids. The amount of antioxidants present at the time of air-curing the tobacco plants is believed to be effective in inhibiting TSNA formation. The objective is to increase the foliar concentration of native antioxidants during senescence and air-curing of burley tobaccos by judicious application of reagents which inhibit TSNA. For burley tobaccos, application preferably occurs between topping of the tobacco plant and harvest.

High concentrations of sodium chloride (NaCl) cause ionic, osmotic and associated secondary stresses to plants. Plant response to those primary and secondary stresses are complex, but can be grouped into three general categories: homeostasis, detoxification and growth control.

Green house grown burley tobacco plants were sprayed with either 0.7% NaCl, 0.35% NaCl, 0.18% NaCl, or control after topping one week before harvest. Once harvested, the leaves were examined to make sure that the leaves were representative of the plant's condition, as discussed above for Example 1. Samples were rapidly separated into the lamina and midribs. The lamina containing portions were rapidly frozen in liquid

nitrogen and either stored at -80°C or analyzed. Total antioxidant capacity was determined by FRAP assay as discussed above for Example 1 and the values were expressed as  $\mu\text{mol/g}$  of fresh weight.

**Table 3**

	<u>Control</u>	<u>0.7% NaCl</u>	<u>0.35% NaCl</u>	<u>0.18% NaCl</u>
5    Total antioxidant capacity ( $\mu\text{mol/g}$ )	148.4	206	229	175

10    While the invention has been described with reference to preferred embodiments, it is to be understood that variations and modifications may be resorted to as will be apparent to those skilled in the art. Such variations and modifications are to be considered within the purview and scope of the invention as defined by the claims appended hereto.